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The Bcl6 Target Gene MicroRNA-21 Promotes Th2 Differentiation by a T Cell Intrinsic Pathway

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Abstract

The transcriptional repressor Bcl6 is a critical regulator of T helper cell fate, and inhibits Th2-type inflammation. We have found that microRNA-21 (miR-21) is a novel target gene for Bcl6 in Treg cells. Bcl6 represses miR-21 transcription through a Stat3 binding element in the promoter, indicating opposing regulation of miR-21 by the two transcription factors. Ectopic expression of miR-21 promoted Th2 differentiation in non-polarized T cells. The pro-Th2 activity of miR-21 was associated with increased Gata3 expression and decreased expression of the miR-21 target Sprouty1. Increased miR-21 promoted Th2 and Treg gene expression in wild-type Tregs. MiR-21 could thus help promote the Th2 bias of Bcl6-deficient conventional T cells and Treg cells. MiR21 expression is increased in Th2-type inflammation, and our results define miR-21 as a critical target of Bcl6, thus providing a new link between Bcl6 and Th2 inflammation. Finally, our results reveal a novel T cell autonomous role for miR-21 in promoting Th2 differentiation.

Keywords

MicroRNA; Bcl6; Th2 differentiation; Regulatory T cells

Introduction

Bcl6 is a potent sequence-specific transcriptional repressor originally identified as an oncogene in B cell lymphoma (Dalla-Favera et al. 1999). *Bcl6*-deficient (*Bcl6−/−*) mice develop severe Th2-mediated myocarditis and pulmonary vasculitis, thus revealing a key role for Bcl6 in inhibiting Th2-type inflammatory disease (Dent et al. 1997; Dent et al. 1998). The spontaneous nature of the inflammatory disease in *Bcl6−/−* mice suggests defects in the regulatory T cell (Treg) lineage, however, little is known about the role of Bcl6 in Treg function. Bcl6 is a lineage-defining transcription factor for follicular helper T cells (Tfh) (Yu et al. 2009). One mechanism for how Bcl6 acts as a master regulator of the Tfh lineage is that Bcl6 represses micro-RNAs that normally repress Tfh cell development (Yu et al. 2009).

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Micro-RNAs (miRs) are important inhibitors of gene expression in multiple biological systems, and function by binding to 3'-UTRs of specific target mRNAs, mediating either mRNA degradation or translational inhibition (Bartel 2004). MiRs are critical for normal cell development and function but are also dysregulated in diseases such as cancer, autoimmunity and inflammation (Cho 2007; Jeker and Bluestone 2010; O'Connell et al. 2012). Individual miRs have been identified that regulate T helper cell differentiation: miR-155 promotes Th1 and Th17 differentiation (O'Connell et al. 2010), miR-326 promotes Th17 differentiation (Du et al. 2009), and miR-29 inhibits Th1 differentiation (Steiner et al. 2011). Mice with a conditional deletion of Dicer in the Treg lineage develop fatal multi-organ autoimmunity similar to Foxp3-deficient mice, revealing a key function for miRs in Treg stability and function (Liston et al. 2008; Zhou et al. 2008). Indeed, specific miRs are differentially expressed in Tregs and regulate Treg cell biology, for instance, miR-155 is required for Treg development (Kohlhaas et al. 2009), miR-142-3p regulates cAMP production in Tregs (Huang et al. 2009), miR-146a regulates ability of Tregs to control Th1-inflammation (Lu et al. 2011), and miR-10a helps stabilize FoxP3 expression in Tregs (Jeker et al. 2012; Takahashi et al. 2012).

MicroRNA-21 (miR21) is the most commonly up-regulated microRNA in a variety of cancers (Jung and Calin 2010). MiR-21 is also increased in allergic disease in both mouse and human (Lu et al. 2009; Lu et al. 2012). MiR-21 can promote Th2 responses by inhibiting IL-12 in myeloid cells (Lu et al. 2009; Lu et al. 2011). While Stat3 and AP-1 can promote miR-21 expression (Loffler et al. 2007; Fujita et al. 2008; Iliopoulos et al. 2010), the signals that regulate miR-21 expression in Th2 inflammation are not understood. MiR-21 is expressed at higher levels in Tregs compared to conventional CD4+CD25− T cells (Cobb et al. 2006), but the functional significance of Treg-specific expression of miR-21 has not been ascertained. Here we report a novel pathway of gene regulation in Treg cells, involving repression of miR-21 by Bcl6, and a novel pathway for promoting Th2 responses via miR-21.

Results

Bcl6 directly represses miR-21 and shares a binding site in the miR-21 promoter with Stat3

MiRs are important regulators of Treg lineage stability under inflammatory contexts (Liston et al. 2008; Zhou et al. 2008), and Bcl6 represses the expression of a large number of miRs in T cells (Yu et al. 2009). We found that *Bcl6−/−* Foxp3$^+$ Tregs express elevated Th2 genes (Gata3 and Th2 cytokines) and fail to suppress Th2 inflammatory responses *in vivo* (Sawant et al. 2012). We thus wondered if Bcl6 regulated Treg lineage stability by repressing miRs in the Treg lineage. MiR profiling of wild-type and *Bcl6−/−* CD4$^+$CD25$^+$Foxp3$^+$ Tregs identified 3 miRs increased significantly in *Bcl6−/−* Tregs relative to wild-type: miR-21, miR-22 and miR-146b (Fig. 1A). Increased expression of these miRs was verified by QPCR analysis (Fig. 1B). To test whether these miRs were regulated intrinsically in Tregs by Bcl6 or extrinsically by inflammatory signals in the *Bcl6−/−* mice, mixed chimeras were generated with bone marrow from CD45.1$^+$ wild-type Foxp3-gfp mice and CD45.1$^-$ *Bcl6−/−* Foxp3-gfp mice, using *Rag1−/−* recipient mice. As shown in Fig. 2, only miR-21 was increased significantly in chimeric *Bcl6−/−* Tregs compared to chimeric wild-type Tregs, indicating that only miR-21 is an intrinsic target of Bcl6 in Tregs, and that miR-22 and miR-146b are not likely to be directly regulated by Bcl6 in Tregs.

To further test the regulation of miR-21 by Bcl6, we infected naïve CD4$^+$ T cells with retrovirus (RV) expressing Bcl6. Ectopic Bcl6 expression resulted in specific repression of miR-21, but not miR-22 or miR-146b relative to control RV (Fig. 3A), consistent with a study by Yu *et al* (Yu et al. 2009), which also noted miR-21 as one of the most strongly down-regulated miRNA by Bcl6 in T cells (~3.9 fold), while miR-146a was down-regulated

(~1.1 fold) and miR-22 was up-regulated (~1.2 fold). Thus, this result confirms miR-21 as a novel Bcl6 target gene in T cells. Next, we analyzed the miR-21 promoter sequence and found two potential Bcl6 binding sites near the start site, which overlap with previously characterized binding sites for the transcription factor, Stat3 (Fig. 3B) (Loffler et al. 2007; Fujita et al. 2008; Iliopoulos et al. 2010; van der Fits et al. 2011; Barnes et al. 2012). Multiple studies have shown that IL-6 or IL-21, acting on Stat3, can positively regulate miR-21 transcription (Loffler et al. 2007; Iliopoulos et al. 2010; van der Fits et al. 2011; Barnes et al. 2012). We found that Bcl6 could repress the wild-type miR-21 promoter significantly in a transient transfection assay in Jurkat T cells (Fig. 3B). Mutation of the 5' Stat3-binding site (SB1) had no effect on repression by Bcl6, whereas mutation of the 3' Stat3-binding site (SB2), completely abolished repression by Bcl6, consistent with SB2 having a better core Bcl6 recognition motif. These data suggested that Bcl6 and Stat3 may compete for regulation of the miR-21 promoter through a common site, with Bcl6 repressing and Stat3 activating miR-21 transcription. To test this further, we analyzed miR-21 induction by IL-6 in CD4 T cells from wild-type, *Bcl6*$^{-/-}$ or *Stat3*$^{-/-}$ mice (Fig. 3C). IL-6 induced miR-21 up to 5.5 fold, whereas in *Bcl6*$^{-/-}$ T cells, basal miR-21 was increased almost 3-fold and induction of miR-21 by IL-6 was augmented over 30-fold. In contrast, miR-21 induction by IL-6 was completely abolished in *Stat3*$^{-/-}$ T cells. Thus, Bcl6 and Stat3 have opposing effects on the transcription of miR-21 in T cells.

MiR-21 promotes Th2 differentiation in a T cell intrinsic manner

MiR-21 can inhibit Th1 responses and promote Th2 responses by repressing IL-12 expression in myeloid cells (Lu et al. 2009; Lu et al. 2011). To assess the functional relevance of miR-21 within T cells, we tested whether miR-21 regulates T cell differentiation in a T cell intrinsic manner. We therefore constructed a miR-21-expressing RV, and infected naïve CD4 T cells from wild-type mice, to test whether over-expression of miR-21 in T cells affected helper cell differentiation activated under non-polarized "Th0" conditions. The miR-21 RV promoted a 3- to 4-fold increase in miR-21 in differentiated T cells (Fig. 4A), which was comparable to the relative increase in miR-21 between wild-type and *Bcl6*$^{-/-}$ Tregs (Fig. 1). In T cells differentiated in the presence of increased miR-21, we observed increases in the Th2 genes, *Gata3* and *Il4*, and a slight decrease in *Ifng* compared to T cells infected with control retrovirus (Fig. 4A). Under Th2 differentiation conditions, miR-21 RV augmented Th2 cytokines but not IFNγ production (Fig. 4B). To further assess miR-21 activity in promoting Th2 differentiation, we tested a synthetic miR-21 "mimic" and a miR-21 "antagomir" (inhibitor) in Th0 differentiation cultures, using a scrambled oligo as control. As shown in Figure 4C, the mimic significantly promoted Th2 cytokine production but not IFNγ production, whereas the antagomir potently inhibited Th2 cytokine production. Thus, miR-21 can specifically promote Th2 differentiation by a T cell autonomous mechanism.

Consistent with the promotion of Th2 differentiation by increased miR-21 expression, miR-21 is expressed higher in Th2 cells compared to Th1 cells (Suppl. Fig. 1). Stat3 is activated during Th2 but not Th1 differentiation (Stritesky et al. 2011), and this Stat3 activity likely promotes the elevated miR-21 in Th2 cells. Furthermore, *Stat3*$^{-/-}$ T cells have defective Th2 differentiation (Stritesky et al. 2011), and a lack of miR-21 up-regulation can account for the reduced Th2 responses of *Stat3*$^{-/-}$ T cells.

MiR-21 can positively regulate Foxp3 expression in human Tregs, although the exact mechanism has not been elucidated (Rouas et al. 2009). Consistent with that observation, we also noted increased Treg genes (Foxp3 and IL-10) following miR-21 over-expression in wild-type Tregs, along-with up-regulation of the Th2 cytokine, Il4 (Fig. 5). Thus, elevated miR-21 expression in Tregs can promote both Treg and Th2 gene expression. This

phenotype is consistent with the gene expression pattern we observed with Bcl6−/− Tregs (Sawant et al. 2012).

MiR-21 target genes in T cells

A large number of miR-21 target genes have been described (Lu et al. 2009; Jung and Calin 2010). To assess miR-21 target genes in $CD4^+$ T cells, we treated cells with miR-21 mimic or scrambled control and analyzed six of the most well characterized target genes (Supp. Fig. 2). Of these six genes, only Sprouty1 (*Spry1*), a negative regulator of the MAP kinase pathway, was consistently decreased by mimic treatment (Supp. Fig. 2, Fig. 6A). Along with decreasing *Spry1*, miR-21 mimic augmented *Gata3* mRNA (Fig. 6A). Since the MAP kinase pathway has been linked to promoting Gata3 protein stability (Yamashita et al. 2005), and Gata3 can auto-activate its own expression (Ouyang et al. 2000), miR-21 may augment Gata3 expression and thus Th2 gene expression by decreasing *Spry1* and increasing MAP kinase activity. We then analyzed miR-21 gene targets in *Bcl6−/−* Tregs (Fig. 6B). We observed a significant decrease of *Spry1* mRNA in *Bcl6−/−* Tregs compared to the wild-type Tregs, indicating that the regulation of *Spry1* by miR-21 occurs in Tregs. The promotion of Th2 differentiation by miR-21 via *Spry1,* may possibly explain the elevated Th2 gene expression exhibited by *Bcl6−/−* Tregs and their failure to control Th2 responses *in vivo* (Sawant et al. 2012). Additionally, Bcl6 repression of miR-21 in Tregs may be required to limit Th2 gene expression in the Treg lineage. Using gene expression from Sawant *et al* (Sawant et al. 2012), we determined the relative expression of 54 potential miR-21 target genes in *Bcl6−/−* Tregs versus wild-type Tregs (Supplementary Table). Of these 54 genes, two were decreased more than 2-fold: *Spry1* and *Satb1*. *Satb1* is known to promote Th2 differentiation (Cai et al. 2006; Ahlfors et al. 2010; Notani et al. 2010), and lower levels of Satb1 do not explain the enhanced Th2-like phenotype of *Bcl6−/−* Tregs. Over 30 other potential miR-21 target genes were decreased in *Bcl6−/−* Tregs by more than 20% compared to wild-type (Supplementary Table), however, a clear immunological role has not been described for the majority of these genes.

Il12a, another reported miR-21 gene target in myeloid cells is involved in Th1/Th2 polarization (Lu et al. 2011). In Tregs, *Il12a* is a component of the Treg suppressive cytokine IL-35 in conjunction with *Ebi3* (Collison et al. 2007). We observed a strong decrease in *Il12a* mRNA in *Bcl6−/−* Tregs compared to the wild-type Tregs (Fig. 6B). The decrease in *Il12a* mRNA in *Bcl6−/−* Tregs may lead to less production of IL-35, which is important in control of Th2 responses in a dust-mite allergen challenge model (Huang et al. 2011). One interesting possibility is that reduced IL-35 secretion of *Bcl6−/−* Tregs factors into their inability to suppress Th2 allergic airway inflammation *in vivo*. Thus, one target gene of miR-21 in Tregs encodes part of an important immunosuppressive cytokine that may be critical for Treg control of Th2 immune responses.

Discussion

Cytokines such as IL-6 and IL-21 are thought to up-regulate miR-21 via Stat3 activation during inflammation, cellular differentiation or growth and survival responses (Loffler et al. 2007; Iliopoulos et al. 2010; van der Fits et al. 2011; Barnes et al. 2012). Here we have found that Bcl6 represents a counter-balance to up-regulation of miR-21 by Stat3. This pathway may be particularly important in regulatory T cells, which are critical for controlling inflammation. Intriguingly, Stat3 and Bcl6 appear to bind to a common site in the miR-21 promoter, indicating the potential for competitive regulation of miR-21 by these factors in cells in an inflammatory environment. Our results imply that T cells within an inflammatory environment can undergo Th2 differentiation due to miR-21 up-regulation, and that this is enhanced in the absence of Bcl6. The deregulation of miR-21 in *Bcl6−/−* T cells could augment Th2 differentiation, and lead to deregulated Th2 inflammation as is seen

in *Bcl6*−/− mice (Dent et al. 1997; Dent et al. 1998). Since miR-21 regulates Th2 differentiation by a separate pathway than the canonical pathway of IL-4 and Stat6, our findings may explain why Th2-type inflammation occurs in *Bcl6*−/− mice independently of IL-4 and Stat6 (Dent et al. 1998). In fact, the Th2-type inflammation in *Bcl6*−/− mice is driven, in part, by IL-6 produced by myeloid cells (Mondal et al. 2010; Ohtsuka et al. 2011). Higher levels of IL-6 in *Bcl6*−/− mice could lead to higher miR-21 in CD4 T cells, which would pre-dispose the T cells to Th2 differentiation.

Stat3 is required for full Th2 differentiation (Stritesky et al. 2011), and the failure to up-regulate miR-21 can explain the incomplete Th2 differentiation seen with *Stat3*−/− T cells. The counter-regulation of miR-21 by Bcl6 and Stat3 can potentially explain the opposing roles of these transcription factors in Th2 differentiation, with Bcl6 inhibiting and Stat3 promoting Th2 differentiation, respectively. Deregulation of miR-21 in *Bcl6*−/− Treg cells can also explain the inability of these cells to control Th2 inflammation (Sawant et al. 2012). The regulation of miR-21 by Bcl6 may also relate to the specific development of myocarditis in *Bcl6*−/− mice, as increased miR-21 expression has been associated with cardiac hypertrophy (Thum et al. 2008).

Curiously, while four other groups have studied the regulation of miR-21 by Stat3, these studies have all relied on chromatin immuno-precipitation of the miR-21 promoter as an indirect test of Stat3 activity (Loffler et al. 2007; Iliopoulos et al. 2010; van der Fits et al. 2011; Barnes et al. 2012). Only one of these studies tested Stat3 function on miR-21 transcription: siRNA was used to knock-down Stat3, resulting in loss of miR-21 expression in mammary epithelial cells (Iliopoulos et al. 2010). In our current study, we show genetic ablation of Stat3 in T cells results in loss of miR-21 induction by IL-6. While none of these other studies used a luciferase assay to show Stat3 activating the miR-21 promoter, we were unable to activate our miR-21 promoter luciferase construct in our T cell system with IL-6. Thus, there may be some unusual regulation of the miR-21 promoter by Stat3, which may be worthy of further investigation.

MiR-21 has been shown to inhibit Th1 differentiation and promote Th2 differentiation by acting in myeloid cells and inhibiting the expression of IL-12 (Lu et al. 2011). Here, we show that miR-21 promotes Th2 differentiation by a novel pathway, in a T cell autonomous manner. One possible mechanism for how miR-21 can promote Th2 differentiation is by inhibiting the expression of the transcript for *Spry1*, a MAP kinase pathway inhibitor. Since the transcriptional activity of the master Th2 factor Gata3 can be stabilized by MAP kinase activity (Yamashita et al. 2005), we propose that increased miR-21 decreases *Spry1*, leading to more MAP kinase activity and increased Gata3 expression. We observed that miR-21 represses *Spry1* transcript in conventional T cells about 1.25-fold, and 1.5- to 2-fold in Treg cells (Figure 6; Supplementary Table). Although these changes in expression are relatively slight, this level of transcript decrease is typical for microRNAs, which fine-tune gene expression via relatively small changes in transcript levels rather than by an on-off mechanism (O'Connell et al. 2012). Given the potential of miR-21 to affect the MAP kinase pathway and Gata3 expression, the regulation of miR-21 by Bcl6 might also explain the post-transcriptional control of Gata3 and Th2 differentiation by Bcl6 (Kusam et al. 2003). In this earlier work, we observed that high levels of Bcl6 could lead to decreased Gata3 protein without affecting Gata3 transcript. Repression of miR-21 by Bcl6 could lead to higher Sprouty1, less MAP kinase activity and decreased Gata3 protein stability.

MiR-21 is specifically increased in Tregs versus conventional T cells (Cobb et al. 2006; Rouas et al. 2009), suggesting that miR-21 may have an important role in Treg biology. MiR-21 is up-regulated in human Tregs, and can positively regulate Foxp3 expression (Rouas et al. 2009). Since many Treg genes are dependent upon Foxp3 for their expression,

regulation of miR-21 by Bcl6 can control expression of Foxp3 and thus other Treg genes. IL-10 is a well-characterized BCL6 target gene in T cells (Kusam et al. 2003) and is positively regulated by miR-21 (Sheedy et al. 2009). Thus Bcl6 may regulate IL-10 indirectly in Tregs via miR-21 regulation. MiR-21 can also decrease *Il12a* expression in Tregs, leading to less secretion of the immune-suppressive cytokine IL-35, which may be important for Treg control of Th2 responses. The constellation of gene changes mediated by increased expression of miR-21 can potentially explain the unusual gene signature of *Bcl6−/−* Tregs with augmented Treg and Th2 genes resulting in potent suppression of T cell proliferation *in vitro* yet exacerbated Th2 inflammation *in vivo* (Sawant et al. 2012).

While miR-21 function has been extensively studied in cancer, the function of miR-21 in T cells and inflammation has not been well characterized. To our knowledge, this is the first study identifying miR-21 as a novel target gene for Bcl6 in Treg cells, and reporting a T cell autonomous role for miR-21 in promoting Th2 differentiation. Finally, we have identified miR-21 target genes that shed light on the role of miR-21 in conventional T cells and Tregs.

Materials and Methods

Mice

Bcl6−/− mice on a mixed C57BL/6-129Sv background (Dent et al. 1997; Dent et al. 1998) were used between 5 and 10 weeks of age, and the mice used were active and relatively healthy. Foxp3-gfp, *Rag1−/−* and BoyJ mice were obtained from the Jackson Laboratory (Bar Harbor, ME). CD4-specific *Stat3−/−* mice have been described (Stritesky et al. 2011). Mice were bred under specific pathogen-free conditions at the laboratory animal facility at IUSM and were handled according to protocols approved by the IUSM Animal Use and Care Committee.

MiR profiling and qRT-PCR assessment of miRs

RNA was extracted from FACS-sorted $CD4^+CD25^+Foxp3^+$ Tregs from wild-type and *Bcl6−/−* Foxp3gfp mice following 16 hr activation *in vitro* with anti-CD3 (5 μg/ml) and anti-CD28 (10 μg/ml). The samples were hybridized to the miRCURY™ LNA array version 11.0 (Exiqon, Denmark), which tests for all miRNAs registered in the miRBASE version 13.0 at the Sanger Institute. Validation of miR expression was performed using TaqMan miR assays (Applied Biosystems). MiRs sno202, sno234 and U6 were used as controls, with U6 as the sole control for samples with limiting RNA.

Generation of Bone Marrow Chimeras

Bone marrow (BM) cells (5×10^6 each type) from donor wild-type BoyJ ($CD45.1^+$) and *Bcl6−/−* ($CD45.1^-$) mice (each on Foxp3gfp background) were injected i.v. into *Rag−/−* recipient mice, sub-lethally irradiated (350 Gy) 24 hrs prior. The lymphoid compartment in the recipients was allowed to reconstitute for 4–5 months. Mice were immunized with OVA/Alum i.p. 2 weeks prior to FACS sorting of the $CD45.1^+$ wild-type and $CD45.1^-$ *Bcl6−/−* $CD25^+Foxp3^+$ Tregs for miR assessment.

Reporter assays

Full-length, SB1 and SB2 miR-21 promoter constructs (1 μg each) and control and Bcl6 expression constructs (1 μg CXN or 0.8 μg CXN plus 0.2 μg CXN-Bcl6 for the respective conditions) were transiently transfected into 1×10^6 Jurkat T cells with X-tremeGENE HP DNA transfection reagent (Roche), according to the manufacturer's protocol. Luciferase measurements were performed 24 hrs after transfection following 6 hr activation of cells with PMA (10 ng/ml) and Ionomycin (0.3 μM) using Luciferase Assay System (Promega).

Cloning of mmu-miR-21 and plasmid construction for miR-21 reporter vectors

The miR-21 gene representing the primary transcript (~300 bp) was PCR amplified from mouse genomic DNA and cloned into a retroviral vector co-expressing $H2K^k$. Full-length mouse miR-21 promoter region was PCR amplified from mouse genomic DNA and was inserted using *Mlu-I* and *Xho-I* restriction enzyme sites into the pGL3-basic vector (Promega). The control retrovirus expresses a leader transcript plus the internal ribosomal entry site and $H2K^k$.

Mouse T helper cell differentiation assays and retroviral transductions

Naïve T cells ($CD4^+CD62L^+$) were purified from lymph nodes and spleen using the MACS system (Miltenyi Biotech). Naïve $CD4^+$ T cells were activated with plate-bound anti-CD3 (5 μg/ml; 145-2C11) and anti-CD28 (10 μg/ml; 37.51) and cultured under standard Th0 and Th2 differentiation conditions (Mondal et al. 2010). Retroviral transductions of T cells were performed as described (Mondal et al. 2010).

MiR-21 mimic and inhibitor

Naïve T cells activated under Th0 conditions were treated with 1 μ M control (scrambled oligo), miR-21 mimic (double stranded RNA oligo) and antagomiR (single stranded DNA oligo) (Exiqon). The oligos were cholesterol linked that enable efficient delivery into cells, without the need of any transfection protocol. Gene expression was assessed 16 hrs following treatment. For assessment of cytokine productions, cells were treated with the oligos over a 5-day period, following which the cultures were re-stimulated with anti-CD3 (10 μg/ml) overnight to obtain supernatants for ELISA.

Gene expression and Cytokine Secretion analysis

RNA isolation, cDNA synthesis, QPCR assays, T cell stimulations and ELISAs were performed as described (Mondal et al. 2010).

Statistical analysis

p values were calculated using Students' T-test. A p value < 0.05 was considered to show a significant difference.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

Treg	Regulatory T cell
miR	MicroRNA
Tfh	follicular helper T cells
RV	retrovirus

Spry1 Sprouty1

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Highlights

microRNA-21 (miR-21) is a novel target gene for Bcl6 in Treg cells

Bcl6 represses miR-21 transcription through a Stat3 binding element in the promoter, indicating opposing regulation of miR-21 by Bcl6 and Stat3

Expression of miR-21 promoted Th2 differentiation in non-polarized T cells by a novel intrinsic pathway

The pro-Th2 activity of miR-21 was associated with increased Gata3 expression and decreased expression of the miR-21 target Sprouty1

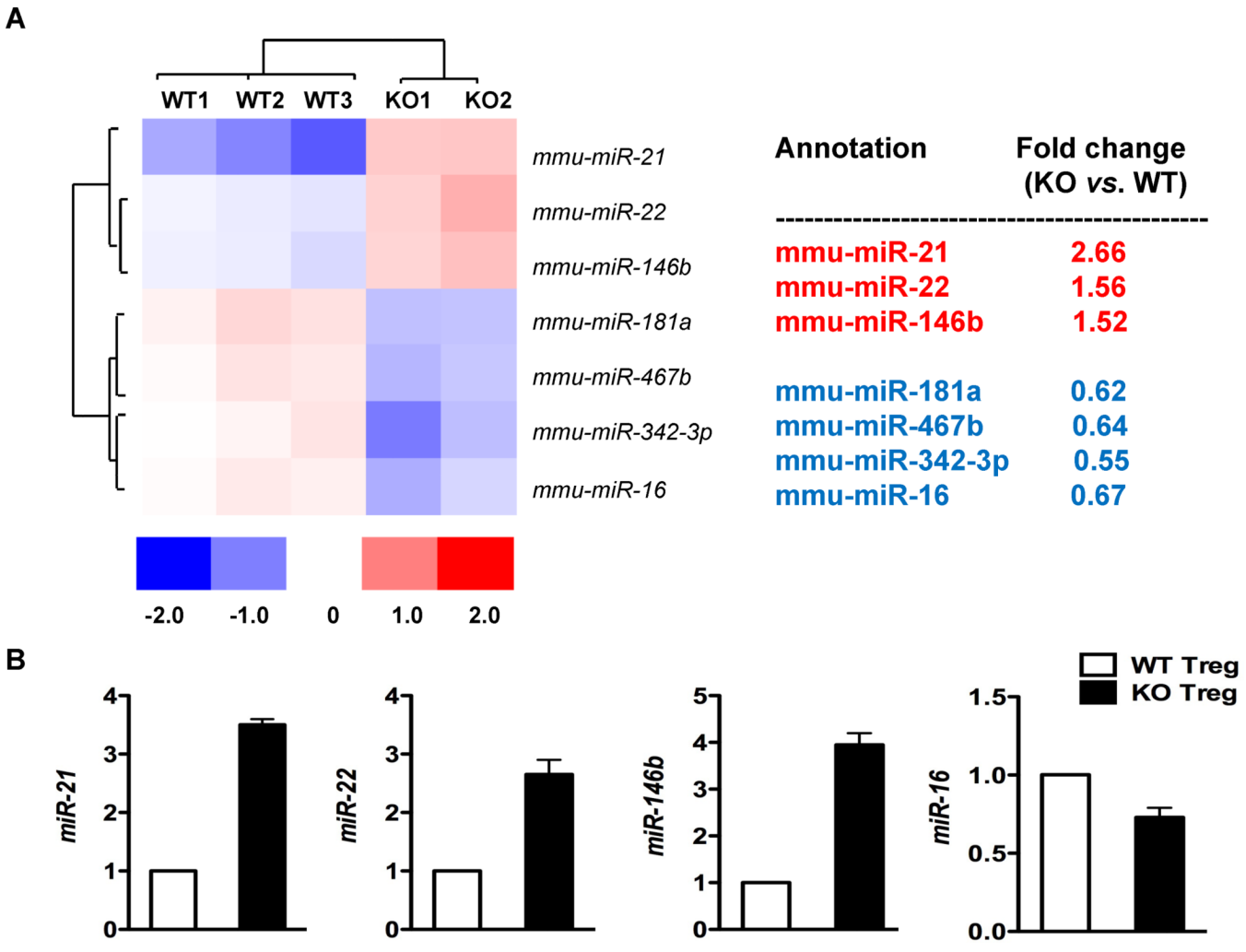

Figure 1. Bcl6 represses miR-21 in Tregs

A. The heat map shows miRs differentially expressed with statistical significance between sorted *Bcl6−/−* and wild-type Tregs, analyzed by expression microarrays. The color scale shown at the bottom illustrates the relative expression level of a miR across all samples: red color represents an expression level above mean, blue color represents expression level lower than the mean. N = Tregs from 3 wild-type mice and 2 *Bcl6−/−* mice.

B. Validation of miR expression in *Bcl6−/−* and wild-type Tregs by QPCR assay. N= Tregs from 2 wild-type mice and 2 *Bcl6−/−* mice.

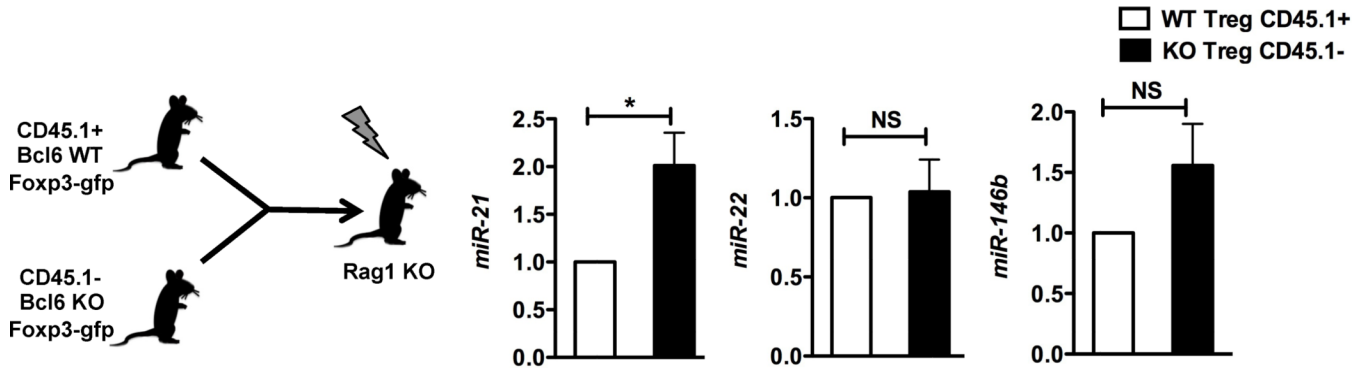

Figure 2. Intrinsic regulation of miR-21 by Bcl6 in Tregs

QPCR analysis of miR transcripts in sorted bone marrow chimera-derived *Bcl6−/−* ($CD45.1^-$) and wild-type Tregs ($CD45.1^+$), following activation for 16 hrs with anti-CD3 and anti-CD28. N= 6 mice per group.

*p<0.05 (Student's t-test) (error bars, s.e.m.)

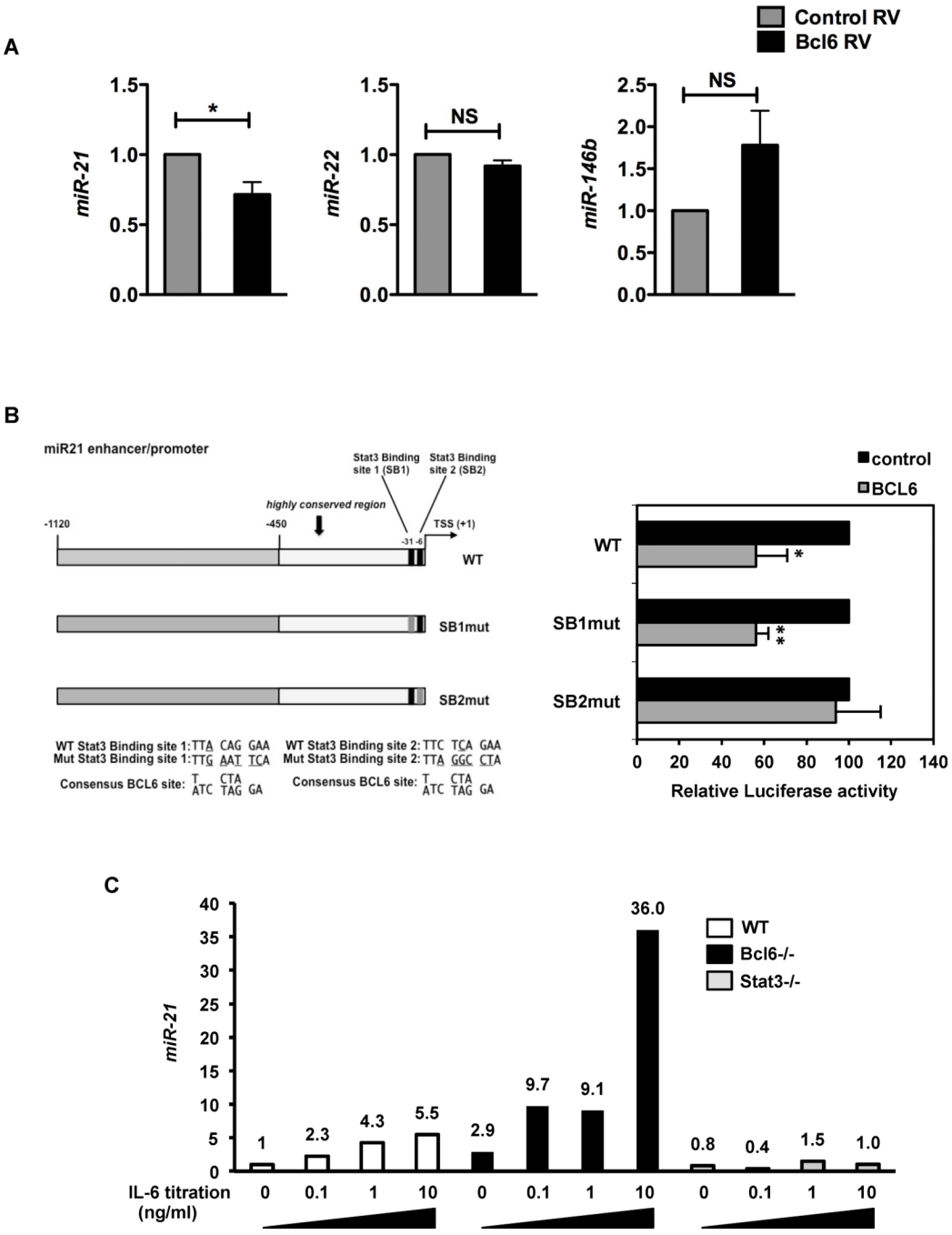

Figure 3. Opposing regulation of miR-21 by Bcl6 and Stat3

A. QPCR analysis of expression of miRs – 21, 22 and 146b following ectopic expression of Bcl6 in naïve T cells. Sorted RV^+ T cells were re-stimulated for 6 hrs to assess its effect on miR expression. Data are averaged from at least 3 different experiments.

B. Luciferase activity in Jurkat T cells co-transfected with full-length or SB1 and SB2 mutated miR-21 promoter driven luciferase reporters and expression constructs for CXN and CXN-Bcl6. Cells were stimulated with PMA and Ionomycin for 24 hrs prior to harvest and luciferase measurement. Results are averaged from 5 independent experiments, where the basal luciferase activity of each promoter construct was set to 100%.

C. QPCR analysis of miR-21 expression following culture of WT, *Bcl6*−/− and *Stat3*−/− naïve T cells ($CD4^+CD62L^+$) in response to increasing doses of IL-6 (0, 0.1, 1, 10 ng/ml) for 24 hrs, with expression normalized to U6. Data representative of 2 independent experiments.
(A–B) *p<0.05, **p<0.01 (Student's t-test) (error bars=s.e.m.)

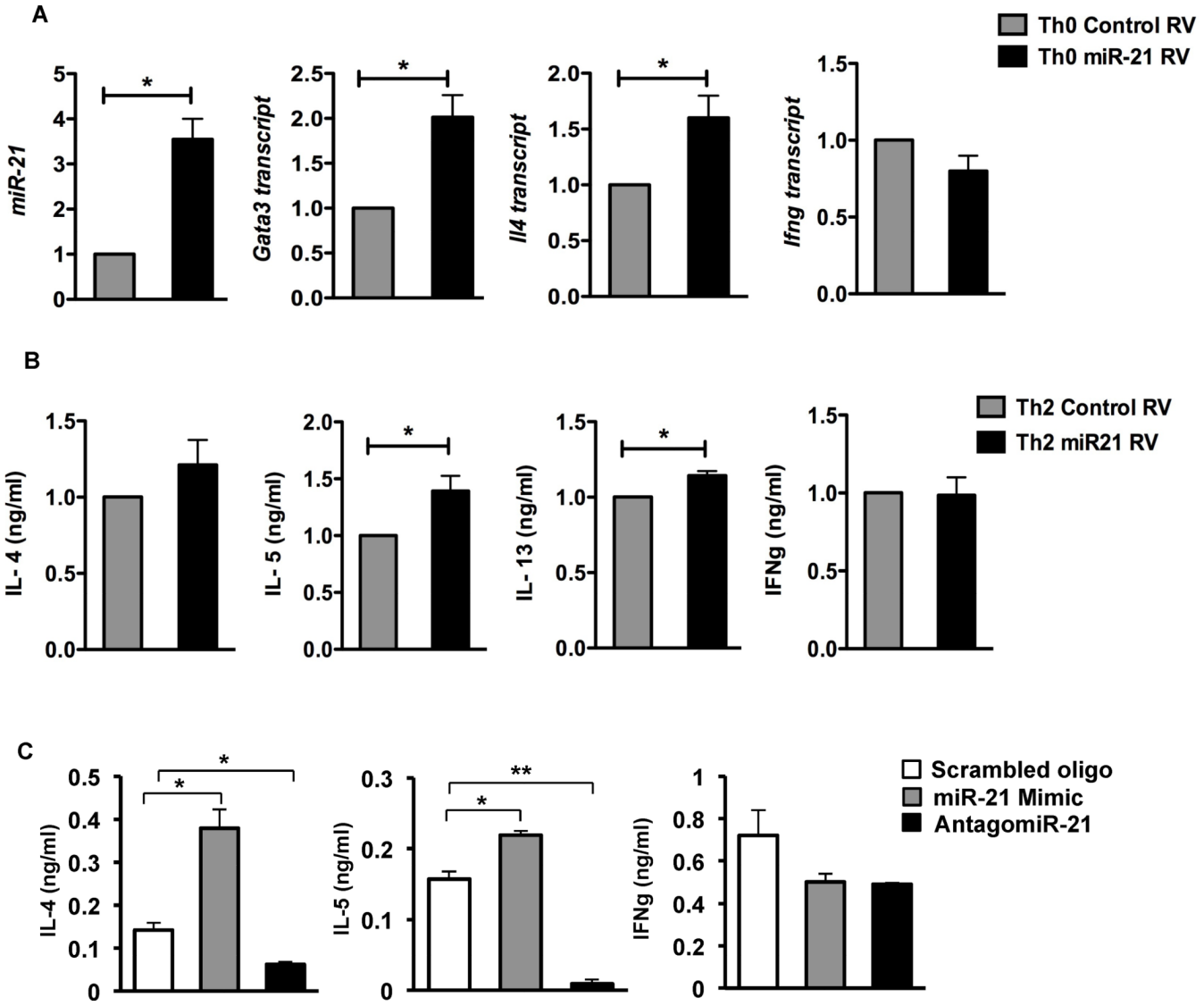

Figure 4. MiR-21 promotes Th2 differentiation in a T cell-intrinsic manner

A. QPCR analysis of miR-21 and *Gata3, Il4* and *Ifng* following ectopic expression of miR-21 RV in non-polarized (Th0) T cells, relative to control RV transduced cells. Sorted RV^+ cells were re-stimulated with anti-CD3 and anti-CD28 for 6 hrs for gene expression analysis. Data are averaged from at least 3 independent experiments.

B. ELISA for cytokines assayed from supernatants of miR-21 transduced Th2 differentiated T cells, relative to control RV transduced cells. Cells were re-stimulated with anti-CD3 and anti-CD28 for 24 hrs following sorting of RV^+ Th2 cells for cytokine measurements.

C. ELISA for cytokines assayed from supernatants of scrambled control, miR-21 mimic and antagomiR-21 treated Th0 differentiated T cells. Cells were cultured with the oligos (1 μ M) over a 5-day period, then re-stimulated with anti-CD3 and anti-CD28 overnight for cytokine measurements. Data representative of 3 independent experiments.

(A–C) *p<0.05, **p<0.01(two-tailed Student's t-test) (error bars, s.e.m.)

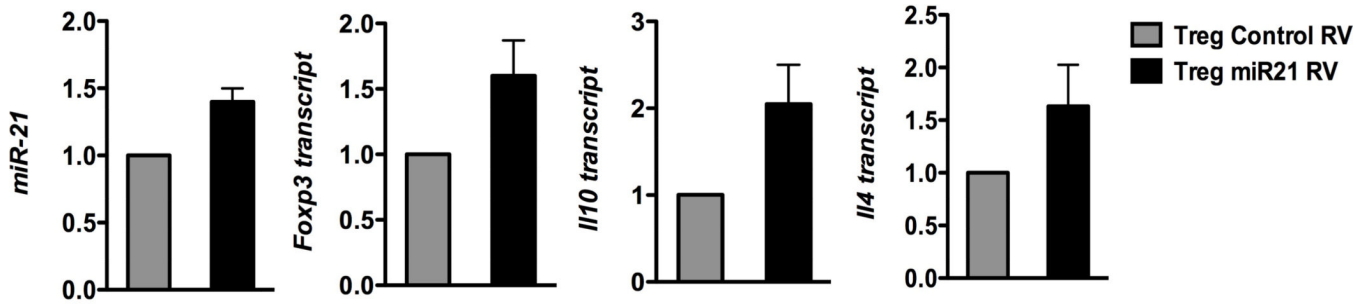

Figure 5. MiR-21 promotes both Treg and Th2 gene expression in Tregs

QPCR analysis of miR-21, FoxP3, Il10 and Il4 in wild-type Tregs transduced with miR-21 RV relative to control RV transduced Tregs. Cells were re-stimulated with anti-CD3 and anti-CD28 for 24 hrs following sorting of RV^+ Tregs for cytokine measurements. Data from both control and miR-21 RV are averaged from 3–4 separate transductions and cell sorts.

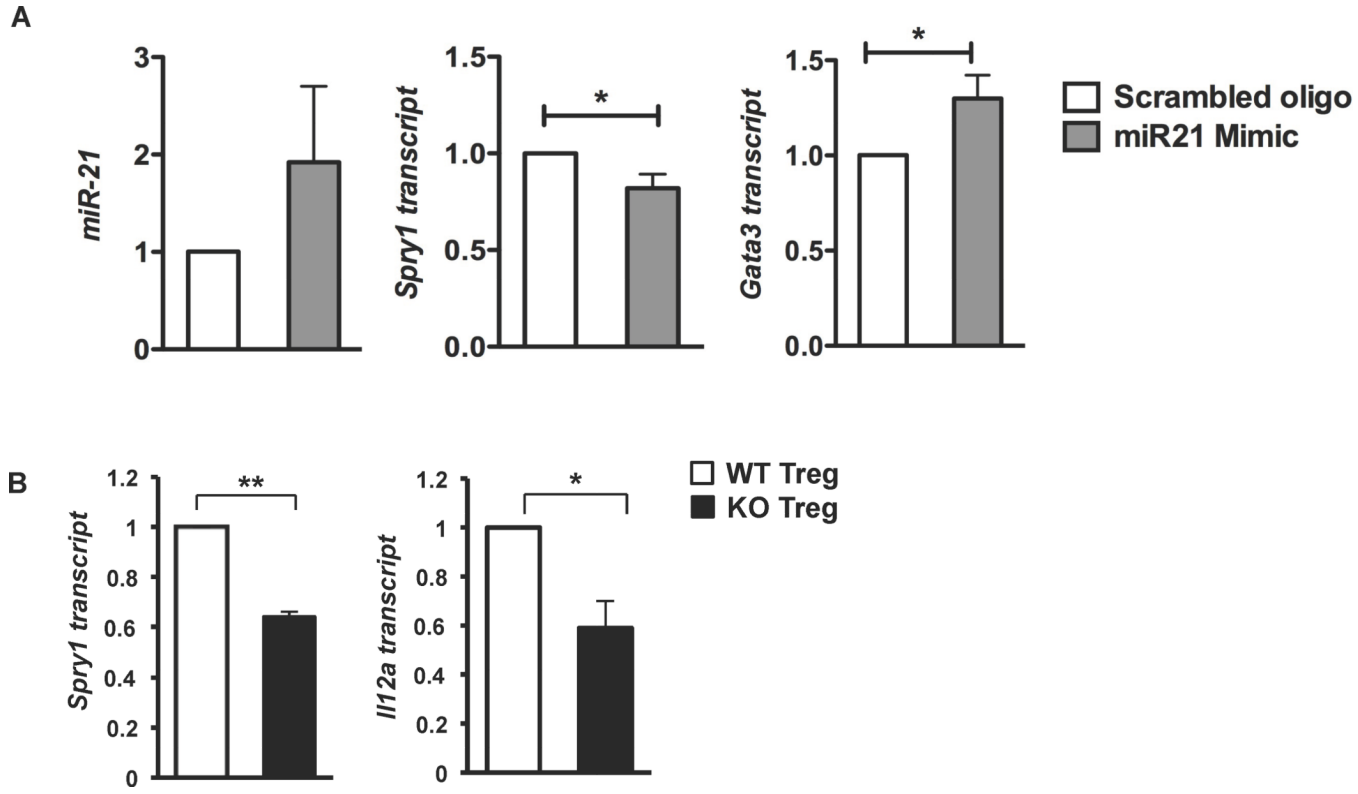

Figure 6. MiR-21 targets *Spry1* and *Il12a*

A. QPCR analysis of miR-21, *Spry1* and *Gata3* following 16 hrs treatment of naïve T cells with scrambled control and miR-21 mimic (1 μ M). Data are averaged from at least 3 independent experiments.

B. QPCR analysis of *Spry1* and *Il12a* in sorted Tregs from *Bcl6*−/−Foxp3-gfp (black bars) and wild-type-Foxp3-gfp mice (white bars). N = 3 mice per group.

*p<0.05, **p<0.01(Student's t-test) (error bars, s.e.m.)